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13. ABSTRACT ***(Maximum 200 Words)***

This study investigates the role of cadherin-11 in the progression of breast cancer from a less invasive, more differentiated phenotype to a more invasive, less differentiated phenotype. The cadherins are transmembrane proteins that have been shown in many previous studies to be involved in cancer progression. Cadherin-11, in particular, is expressed preferentially in breast cancers exhibiting a more invasive, less differentiated phenotype. Results of this study indicate that cadherin-11 expression in MDA-MB 231 breast cancer cells is significantly regulated by β-catenin signaling at both the protein and RNA levels. β-catenin, a signaling molecule in the *wnt-1* pathway, affects transcriptional regulation of gene expression in both colon and breast cancer, including the expression of *c-myc* and cyclin D. In addition, the function of cadherin-11 has been examined by stable transfection of cadherin 11 intact and/or variant into two well-differentiated breast cancer cell lines. We have found that cadherin 11 confers a specific phenotype to normally well-differentiated epithelial cells, including increased invasion. In conclusion, examination of both the regulation and function of cadherin-11 expression may offer clues about the nature of breast cancer progression, especially the processes of invasion and metastasis.

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INTRODUCTION

This study investigates the role of one member of the cadherin family of adhesion molecules, cadherin-11, in the progression of breast cancer from a less invasive, more differentiated phenotype to a more invasive, less differentiated phenotype. The cadherins are transmembrane proteins that mediate cellular interactions via homotypic extracellular association, and have been shown in many previous studies to be involved in cancer progression. Cadherin-11, in particular, was previously shown to be expressed preferentially in breast cancers exhibiting a more invasive, less differentiated phenotype. In this study, our examination of the function and regulation of cadherin-11 in breast cancer was facilitated by the design, production and characterization of monoclonal antibodies raised against the intracellular portion of the protein (in collaboration with Zymed Laboratories). Results indicate that cadherin-11 expression in MDA-MB 231 breast cancer cells is significantly regulated by β-catenin signaling at both the protein and RNA levels. β-catenin, a signaling molecule in the *wnt-1* pathway, is thought to affect transcriptional regulation of gene expression in both colon and breast cancer, and has already been shown to regulate the expression of *c-myc* and cyclin D. We are currently investigating whether the regulation of cadherin-11 expression is indeed at the transcriptional level, and how the *wnt-1* pathway may be related to the regulation of cell adhesion. In addition, the function of cadherin-11 in cancer cells is being examined by stable transfection of cadherin 11 intact and/or variant into SKBR3 cells (lack cadherins) and MCF7 cells (contain E-cadherin). Changes in proliferation rate and motility were assessed by in vitro proliferation assays and Boyden chamber assays. We have found that cadherin 11 confers a specific phenotype to normally well-differentiated epithelial cells, including slightly increased proliferation and significantly increased invasion. In conclusion, we have begun to examine the potential basis of the expression of cadherin-11 in breast cancer cells exhibiting a more invasive, less differentiated phenotype. Examination of both the function and regulation of cadherin-11 expression may offer clues about the nature of breast cancer progression, especially the processes of invasion and metastasis.

BODY

Cell to cell adhesion is a phenomenon often affected in cancer. Important for everything from development to cellular communication, the mechanisms of adhesion may offer clues about the nature of metastasis, invasion, and cancer progression. One important class of cell adhesion molecules is that of the cadherins. These are a family of calcium-dependent transmembrane proteins that mediate cell-cell interactions through homotypic extracellular associations.[1] They are particularly important during cellular differentiation and morphogenesis. Anchoring cadherins to the actin cytoskeleton area a second class of proteins known as catenins, three of which have been identified: α-catenin, similar to the actin-binding protein vinculin; β-catenin, homologous to the Drosophila segment polarity gene Armadillo; and χ-catenin, or plakoglobin, found in adherens and desmosomal junctions.

β-catenin itself is a 92-kD protein that contains several conserved regions known as armadillo repeats.[2] Although originally identified as a link between E-cadherin and the actin-bound α-catenin, recent studies have established β-catenin's role as not only a cell adhesion molecule but also as a signaling molecule in the *wnt-1* pathway, with putative roles in both colon and breast cancer.[3,4]

The *wnt-1* pathway, which is thought to be involved both in normal development and cancer, is still under investigation. It is believed that the *wnt-1* signal indirectly leads to the down-regulation of GSK3β, a serine-threonine kinase. Normally, the absence of the *Wnt* signal allows GSK3β to phosphorylate the APC gene-product, which in turn reduces cytoplasmic levels of β-catenin protein. It is though that APC and GSK3β function in concert to control cytoplasmic β-catenin levels by targeting the protein for

degradation. In the presence of the *Wnt* signal, cytoplasmic β-catenin levels remain high. Many breast and colon cancer cell lines, due to both known and putative mutations in many of the molecules of this pathway, also exhibit high levels of cytoplasmic β-catenin. In addition, recent studies have found that β-catenin interacts with the TCF/LEF family of known transcription factors.[5] This, in addition to evidence that β-catenin accumulates in the nucleus when cytoplasmic levels are increased, has lead to speculation that β-catenin serves to regulate the expression of other gene products which may be important factors in the etiology of cancer at the cellular level.[6]

The goal of this study is to identify putative downstream targets of β-catenin, and to study how these gene products predispose cells to cancerous phenotypes. In order to do this, we attempted to use a two-pronged approach: first, the application of the gene-trap technique; and second, the identification and investigation of several candidate targets, based upon previous studies. One of these, Cadherin-11, is very promising, and has been investigated vigorously.

Cadherin-11 is a member of the family of Type II cadherins, all homotypic cell adhesion molecules related to one another structurally. Originally identified as an adhesion molecule predominantly expressed in tissues of mesodermal origin,[7] cadherin-11 has been subsequently identified as potentially relevant in the aggressive phenotypes of breast, colon, and renal cell carcinoma.[8,9,10] Cadherin 11 exhibits a unique mRNA splice site, allowing for two forms of the protein to be expressed: a normal, intact version (cad 11 INT) and a C-terminus-truncated variant (cad 11 VAR). While the variant has an extracellular domain identical to that of the intact, a frame-shift event at the point of splicing confers a unique cytoplasmic region with absolutely homology to the intact

cytoplasmic domain. The function of the variant protein is unknown, but its sequence does bear some similarity to the src family of tyrosine kinases. In all situations so far examined, cadherin 11 intact and variant forms are expressed co-incidentally[11].

The following results provide preliminary data to demonstrate that β-catenin signaling may play a role in regulating the expression of cadherin-11. In addition, the function of cadherin-11 in cancer cells has been examined by stable transfection of cadherin 11 intact and/or variant into SKBR3 cells (lack cadherins) and MCF7 cells (contain E-cadherin). Changes in proliferation rate, motility, and anchorage independent growth were assessed by in vitro assay, including invasion of extracellular matrix in Boyden chambers, and soft agar growth. We have found that cadherin 11 confers a specific phenotype to normally well-differentiated epithelial cells, including increased proliferation and invasion. In conclusion, we have begun to examine the potential basis of the expression of cadherin-11 in breast cancer cells exhibiting a more invasive, less differentiated phenotype, and the potential regulation of this phenotype by β-catenin. Examination of both the function and regulation of cadherin-11 expression may offer clues about the nature of breast cancer progression, especially the processes of invasion and metastasis.

MATERIALS AND METHODS

Cell Culture and Production of Stable Transfectants SKBR3 cells were obtained from the ATCC (Rockville, MD) and cultured at 37°C, 5% CO_2 in DMEM (Life Technologies, Inc.) plus 10% FBS (Biofluids). Cells were transfected with pCXN2-cad11-intact and/or pCXN2-variant*, or pCDNA3-CAT (as a control) using the calcium

* Both plasmids were gifts from Akira Kudo, Tokyo Inst. of Tech.

phosphate method. Cells were selected in 0.8mg/ml G418 for 4-6 weeks. Cells expressing both forms of cad11 were isolated by FACS sorting and characterized by western blot and/or immunocytochemistry. Alternatively, a second population of SKBR3 cells were cotransfected with pCXN2-cad11intact and/or pCXN2-variant, or pCDNA3-CAT and equal amounts of the puromycin-resistance plasmid pHA262pur**. Resistant pools were subsequently selected in 1ug/ml puromycin for 4-6 weeks and characterized by western blot and/or immunocytochemistry.

MCF7 cells were obtained from the ATCC (Rockville, MD) and cultured at 37°C, 5% CO_2 in DMEM (Life Technologies, Inc.) plus 5% FBS (Biofluids). Cells were transfected with pCXN2-cad11-intact and/or pCXN2-variant, or pCDNA3-CAT (as a control) using Lipofectamine Plus (Life Technologies, Inc.) and selected in 0.5mg/ml G418 for 4-6 weeks. Clonal populations of cells were obtained by plating to a limiting dilution in 0.5 mg/ml G418; subsequent clonal populations were screened by immunocytochemistry and western blot analysis.

Antibodies The following primary antibodies were used for immunocytochemistry, immunoprecipitation, and immunoblotting: monoclonal anti-cadherin 11 clone 5B2H5 and polyclonal anti-cadherin 11 pWTID (Zymed Lab.), both of which were raised against the intracellular domain of cad11 and hence recognize the intact cad11 alone; Monoclonal anti-cadherin 11 (a gift from MJ Bussemakers) and monoclonal anti-cadherin 11 113H (ICOS Corp.), both of which recognize the extracellular domain of both the intact and variant forms of cad11; monoclonal anti-β catenin (Trans. Lab);

** A generous gift from Dr. H. te Riele, Netherlands Cancer Institute, Amsterdam, The Netherlands

polyclonal anti-β catenin SHB7 (a gift from D Rimm); monoclonal anti-pp120^{ctn} (Trans. Labs); and monoclonal anti-p120^{ctn} clone 12F4 (AB Reynolds).

Immunocytochemistry and Microscopy Cells were plated on sterile 18mm glass coverslips and allowed to adhere at least 48 hours prior to fixation in 2% paraformaledyde (20 minutes), with subsequent permeabilization in 0.2% Triton-PBS (5 minutes). Cells were blocked for 1 hour at room temperature in 3% ovalbumin-PBS, then incubated with the appropriate antibody diluted in 6% normal goat serum-PBS for 1 hour room temperature. After 3-3 minute washes in PBS, cells were incubated with the appropriate secondary antibodies conjugated to either Texas Red (Jackson?) or FITC (??) for 1 hour at room temperature in the dark. All secondary antibodies were diluted 1:200. For double-staining, polyclonal primary and anti-rabbit secondary were applied first, followed by monoclonal primary and anti-mouse secondary. After the final 3-3 minute washes in PBS, coverslips were dried and mounted on slides with Vectashield. All fluorescence and Nomarski Interference Contrast images were digitally captured using an Olympus Fluoview Confocal Microscope; brightfield images were digitally captured on a Zeiss Microscope.

Immunoblotting and Immunoprecipitation Cells were solubilized in ice-cold 1% NP-40 buffer solution (1% NP-40, 1250 mM NaCl, 50mM Tris pH 8.0) containing 1mM sodium vanadate, 50mM sodium fluoride, and protease inhibitors (BM...). Lysates were centrifuged at 14,000 rpm for 15 minutes at 4° C to remove the NP-40 insoluble material. After addition of 2X sample buffer (4% SDS, 120mM Tris pH 6.8, 20% glycerol) to the NP-40 soluble fraction, Bio-Rad protein assays were performed to determine total protein content. After addition of reducing agent, the samples were boiled, and equal total

protein was loaded on 3-8% NuPage tris-acetate gels (Invitrogen Inc.) unless otherwise indicated. Proteins were blotted to nitrocellulose (Schleicher & Schuell) and blocked for 1hour at room temperature or overnight at 4° C in 5% milk-PBST. After incubation with appropriate primary and secondary antibodies each for 1 hour at room temperature, blots were treated with ECL reagent (Amersham) and exposed to film. Blots were sometimes stripped (62.5 mM Tris pH 7.5, 2% SDS, 1.7% B-mercaptoethanol for 30 minutes at 50°C), reblocked, and reprobed with new primary and secondary antibody.

For immunoprecipitation, lysates were obtained as described above. Lysates were first precleared with 50 μl Protein G-sepharose beads (Zymed Lab.) alone for 1 hr at 4° C. The beads were spun down, removed, and appropriate precipitating antibodies were added to lysates for 1 hour with rocking at 4° C; 50μl of new beads were then added with rocking for an additional 2 hours at 4°C. After three washes with ice-cold lysis buffer, sample buffer and reducing agent were added to precipitated proteins and beads and boiled for 10 minutes; samples were subsequently analyzed by immunoblotting as described above.

Reporter Assays 12-well plates were seeded at 10^5 cells/well 24-48 hours prior to transfection. Cells were transfected with TOPFLASH reporter (indicates LEF reporter activity), pCXN2-cad11intact and pCXN2-variant or pCDNA3-CAT, and the TK-renilla luciferase plasmid (Promega Inc.) to control for variations in transfection efficiency. After lysis, luciferase and renilla activity were read on a standard luminometer using the Dual-Reagent Luciferase Kit (Promega, Inc.). Luciferase values were normalized to renilla values and plotted relative to control. Each experiment was performed in triplicate at least three independent times, with error bars representing standard deviation.

Proliferation assays WST-1 assays (Boehringer Mannheim) were performed as indicated by the manufacturer. Briefly, 1000-3000 cells of each population were plated in triplicate in 96 well plates on Day –1. WST-1 readings were taken on alternate days beginning with Day 0 on an.... optical densitometer. For analysis (Sigma Plot™), data for each population was plotted relative to the mean Day 0 value to account for variance in plating efficiency, with error bars representing standard deviation. All experiments were performed independently at least three times.

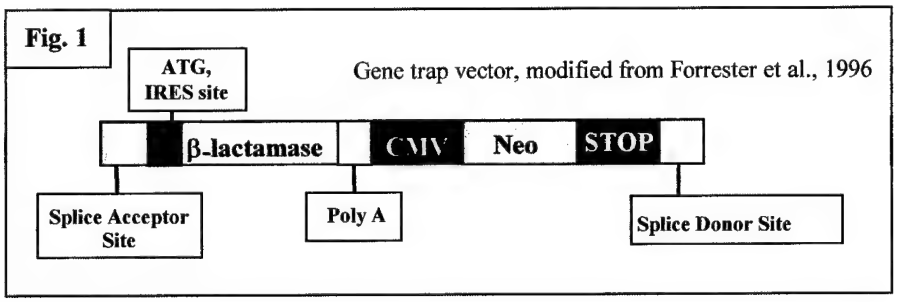
Soft Agar Assays Cells were plated in 6-well plates at 5 x 10^5 cells/well in a 0.3% agar suspension (Sachem...) on a 0.6% agar cushion, with one ml DMEM + 5% FBS covering the cells. The cells were incubated at 37°C + 5% CO_2 and media was carefully changed every 3-4 days. After 2 or 3 weeks colonies greater than 40μm in diameter were scored by an Omnicon 3600 Colony Counter; data was subsequently analyzed and graphed on Sigma Plot™. All experiments were performed in triplicate at least three independent times; error bars represent standard deviation.

***In vitro* Invasion assays** Invasion assays were performed as follows. Standard Boyden chambers were prepared by placing NIH3T3-conditioned media (24 hours, DMEM + 50μg/ml ascorbic acid) in the bottom well of the chamber as a chemoattractant. After coating a 12-μm pore size polycarbonate filter (Poretics, Inc.) with 10ug matrigel, 3x10^5 cells in DMEM with 0.1% BSA were placed in the upper chamber and incubated for 16 hours at 37°C. Membranes were then removed, cells fixed in 25% methanol with 0.5% crystal violet, and remaining cells wiped from the top side of the membrane with a damp cotton swab. Quantification of cells on the bottom of the membrane was performed by

counting the number of cells/field in five random fields per membrane; the fields were then averaged. Bars represent the mean of each population over three membranes with error bars representing standard deviation. Each experiment was performed at least three independent times

RESULTS

Gene Trap. As explained in the previous update, the original gene trap vectors provided to us by LM Forrester[12] contained the *lacZ* reporter gene.



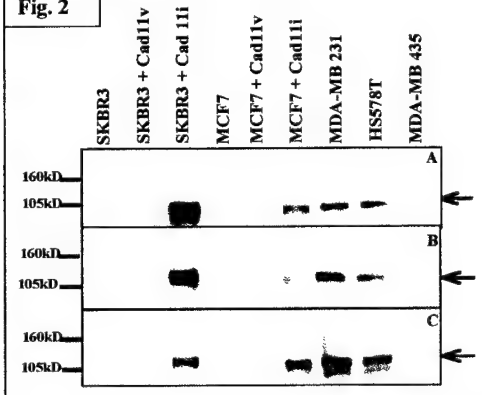
Reengineering of this vector (Figure 1) was performed in collaboration with Dr. Robert Lechleider[] with the following results: insertion of an IRES site to minimize difficulties pertaining to potential internal ATG's in the trapped gene, use of a splice donor site to facilitate vector insertion, as well as a terminal stop codon. Most importantly, the *lacZ* reporter cassette was replaced with β-lactamase, which is a much more sensitive detection agent than GFP.[13] While cells were to be stably transfected and subsequently screened, the time involved and technical difficulties concerning construction of this vector have caused this project to take more time than anticipated, resulting in the additional pursuit of the cadherin-11 studies as follows. Pursuit of gene-trap technology will hopefully be fulfilled by another member of our laboratory or a collaborator, as we

[] Department of Pharmacology, Uniformed Services University of the Health Sciences; Bethesda, MD

decided to pursue the cadherin 11 data, which appeared to be yielding interesting information in a more timely manner.

Cadherin-11 As noted in the introduction, it has been previously published that

Fig. 2



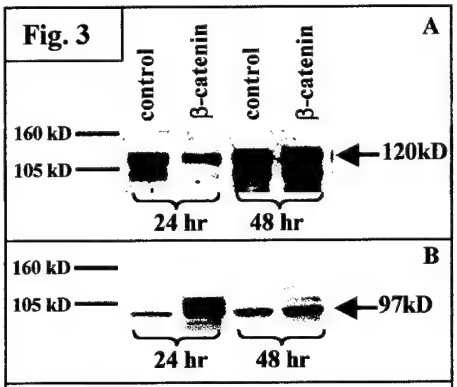
Antibody Characterization

Characterization of Antibodies 2F9D9 (A), 5B2H5 (B), and 3D2G8 (C), all monoclonal antibodies raised against intracellular portions of human Cadherin-11 (intact). SKBR3 cells, which do not express endogenous cadherins, including 11, and MCF7 cells, which express E-cadherin but not Cadherin-11, were transfected with both the intact and variant forms of the protein (the variant form has a different intracellular domain, and therefore should not be recognized by these antibodies. Additionally, MDA-MB 231cells express only Cadherin-11, while HS578T cells express Cadherin-11 and N-cadherin. Finally, MDA-MB 435 cells express only N-cadherin. After NP-40 lysis, soluble fractions were separated by gel electophoresis, transferred to nitrocellulose, and probed with the appropriate antibodies at 1:1000 dilutions.

Cadherin-11 is of significant interest in breast cancer. In addition, previous research has suggested that β-catenin may contribute to the regulation of its expression. [14] In order to facilitate detailed analysis of β-catenin's possible role, antibodies to the intracellular portion of cadherin-11 were designed and produced in collaboration with Zymed Laboratories (Figure 2). After careful characterization, monoclonal antibody 5B2H5 was selected for use during experiments.

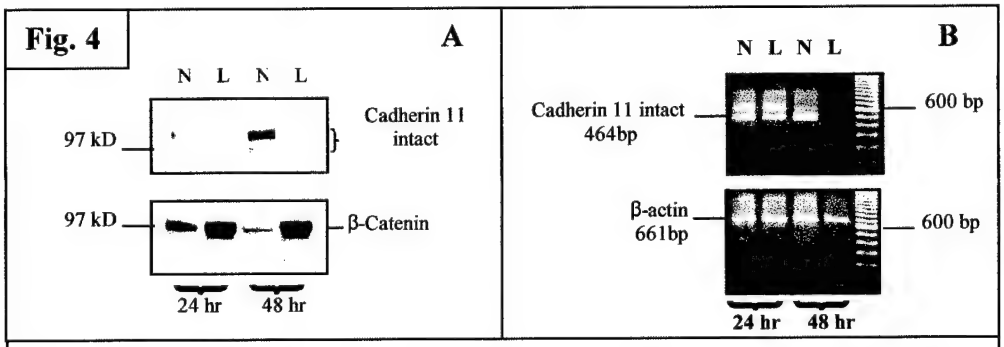
In order to investigate β-catenin's ability to regulate cadherin-11 expression, MDA-MB 231 cells, which normally make cadherin-11, were transiently transfected with a FLAG-tagged β-catenin expression vector, and cadherin-11 expression was analyzed. Figure 3 illustrates the resulting down-regulation of cadherin-11 protein. Preliminary experiments during which cells were treated with Lithium, an inhibitor of GSK3β, yield similar results on the protein as well as RNA level (Figure 4). Further insight into this

regulatory phenomenon will hopefully expand current knowledge of β-catenin's role as an oncogenic signal transducer.



$8x10^5$ MDA-MB 231 cells were transfected with FLAG-tagged β-catenin or pcDNA-CAT (control)and lysed with NP-40 at 24 and 48 hours post-transfection. Equal amounts of total soluble protein were loaded and separated by gel electrophoresis, and subsequently transferred to nitrocellose. A) Cadherin-11(120kD) is decreased at 24 hours. At 48 hours, as β-catenin levels return to normal, cadherin-11 levels do the same. B) The same blot stripped and reprobed for β-catenin (97kD). A larger species, the FLAG-tagged protein, is also picked up by the β-catenin antibody.

While pursuing our investigation of the regulatory elements concerning cadherin 11, we also attempted to address the functional roles it may play in carcinogenesis and invasion of breast cancer. In order to do this, we stably transfected cadherin 11 INT and/or VAR into SKBR3 cells, which lack any known cadherins, and into MCF7 cells, which express E-cadherin. Both cell lines are well-differentiated breast cancer cells lines, minimally invasive and relatively poorly metastatic. Upon expression of either cadherin INT and/or VAR or a control plasmid, we investigated the

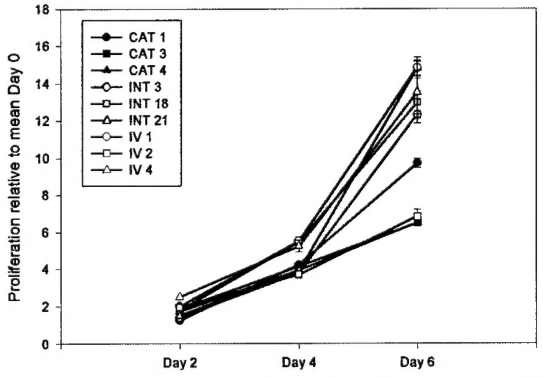


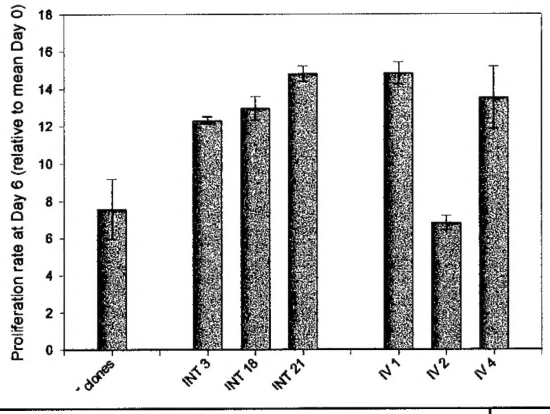
10^6 MDA-MB 231 cells were seeded in 10cm dishes and subsequently treated for 24 or 48 hours with 20mM LiCl or 20mM NaCl (control) + 10mM inositol. After treated, cells were lysed and either protein (A) or total RNA (B) was isolated. A) Equal amounts of total NP-40 soluble protein were loaded and separated by gel electrophoresis and subsequently transferred to nitrocellulose. At 24 hours and more dramatically at 48 hours, cadherin 11 levels decrease in the LiCl treated cells. LiCl, in the presence of inositol, is a specific inhibitor of GSK-3β, which normally targets β-catenin for degradation. Inhibition of GSK-3β leads to an increase in β-catenin, as seen on the blot below, which was stripped and reprobed for β–catenin. B) Semi-quantitative RT-PCR shows the same pattern for mRNA levels; by 48 hours, esp., cadherin 11 mRNA is practically gone. β-actin was used as an internal control for equal mRNA loading.

carcinogenic potential of these cells by examining proliferation rate and the ability to invade an extracellular matrix.

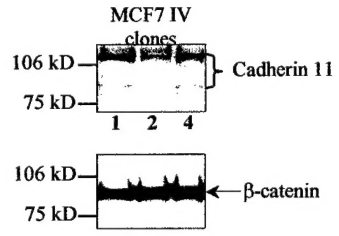
Expression of cadherin 11 in MCF7 cells appears to confer a phenotype that includes an increased proliferation rate (Figure 5). MCF7 cells expressing cadherin 11 grew at approximately twice the rate of control cells with the exception of one

Fig. 5



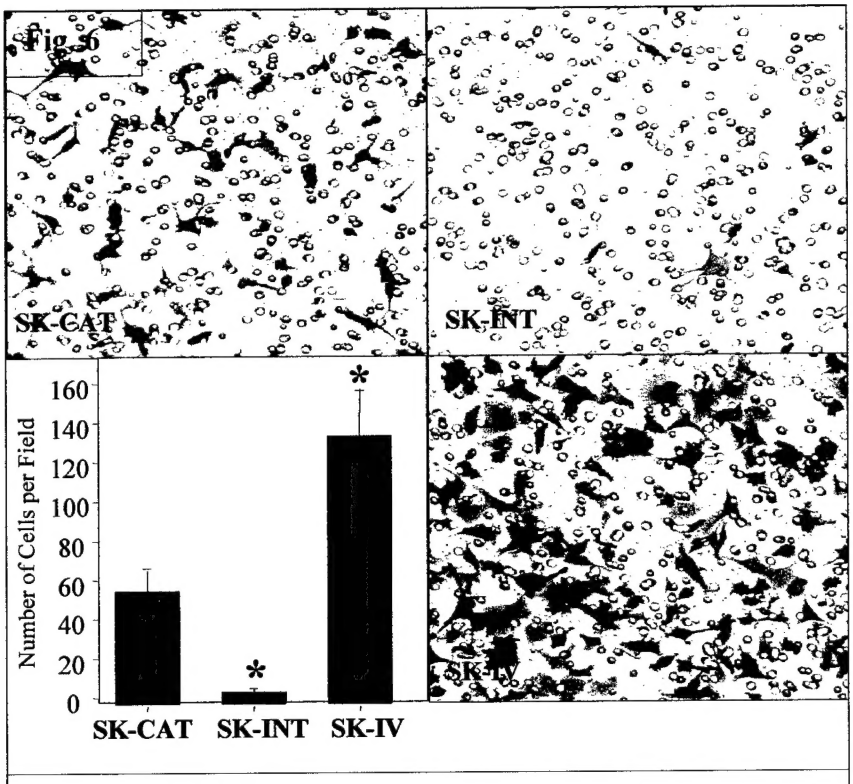


MCF7 cells with and without cadherin 11 were analyzed with respect to proliferation rate using WST-1 (Boehringer Mannheim) according to manufacturer's instructions. A) Proliferation assay results. B) Proliferation at day six expressed in bar form. Not that the IV clone 2, which does not exhibit increased proliferation, expresses less cadherin 11 (C).



experimental clone; upon further examination it was found that this clone had a decreased expression of cadherin 11. Increased proliferation may be one way by which cadherin 11 might cause normally well-differentiated cancer cells to act in a more aggressive, tumorigenic manner.

Cadherin 11 expression also appeared to increase the ability of SKBR3 cells to invade an extracellular matrix in a standard Boyden chamber assay (Figure 6). Preliminary results indicate, interestingly, that expression of the intact form of cadherin



The invasive potential of SKBR3 cells expressing CAT-control, cadherin 11 INT, or IV (INT + VAR), were studied using a standard Boyden chamber assay as described in Methods. Note that cad 11INT cells barely migrated through the membrane compared to control, while those expressing both forms migrated approximately twice as well (* indicates significant difference compared to control by *t*-test).

11 alone actually decreases invasiveness compared to control cells; introduction of the variant form, however, increases invasiveness to a level approximately two-fold over control. These data were confirmed in MCF7 cells in parallel experiments (data not shown). The results indicate that cadherin 11 may indeed be a marker, and possibly a causal factor, for increased invasive potential in breast cancer cells. Future studies will continue to investigate these exciting findings, focusing particularly on the interesting role that cadherin-11 variant may play in this system..

KEY RESEARCH ACCOMPLISHMENTS:

- Construction of new gene-trap vector
- Preparation of cell lines (i.e. stable transfection) with gene-trap vector
- Design and production of specific monoclonal antibodies against cadherin-11
- Analysis of cadherin-11 profile in invasive breast cancer cells
- Data supporting β-catenin's role in regulation of cadherin-11 expression
- Preparation of two stable cell lines (SKBR3, MCF7) expressing cadherin 11 INT and/or VAR
- Analysis of above cells lines, including any changes in invasive or proliferative phenotype

REPORTABLE OUTCOMES

- Development of specific monoclonal antibodies against cadherin-11 (in collaboration with Zymed Laboratories). These antibodies are now commercially available.
- Completion and Defense of Doctoral Thesis: "Cadherin-11 in Breast Cancer," November 17th, 2000.
- Second author manuscript:
  - Pishvaian, MJ, CM Feltes, P Thompson, MJ Bussemakers, JA Schalken, and SW Byers. 1998. Cadherin-11 is expressed in invasive breast cancer cell lines. *Canc. Res.* 59:947-952.

CONCLUSIONS

These studies have resulted in evidence that β-catenin may indeed regulate the expression of cadherin-11 in breast cancer cells. Further, we have shown that this regulation also occurs at the RNA level. Further studies important to this work should include investigation of whether or not this is a transcriptional or post-transcriptional event, as well as isolation and characterization of the cadherin-11 promoter.

In addition, we have begun to address the functional studies pertaining to cadherin-11's role *in vivo*. We have constructed stable cells lines expressing cadherin 11 and studied the effects this exogenous expression has on cellular behavior. We have found that this expression not only results in modestly increased cellular proliferation, but also in an increased ability to invade the extracellular matrix. Both events indicate that

these cells, upon expression of cadherin 11, may indeed take on a more aggressive, invasive phenotype. Further studies should include additional *in vitro* characterization of these cells, including investigating the individual roles of cadherin 11 intact and variant, as well as later *in vivo* studies, such as determining whether or not expression of cadherin 11 allows for increased metastasis of these cells in nude mice.

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